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Suitability of serum cytokine profiling for early diagnosis of implant-associated infections after orthopaedic surgery: A preliminary prospective study

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ABSTRACT

The C-reactive protein (CRP) is still the conventional marker used to diagnose implant-associated infections (IAI) after orthopaedic surgery. However, the CRP level can lead to misdiagnosis since it is up-regulated not only during bacterial infection. In this prospective study, we evaluated the serum cytokine profile before (pre-OP) and after orthopaedic surgery (post-OP) as well as after confirmation of a developed infection (COI) to identify candidate biomarkers for diagnosis of IAI. Sera from 10 controls 7 to 1 days pre-OP and 0 to 22 days post-OP as well as from 5 patients who developed IAI 5 to 1 days pre-OP, 0 to 197 days post-OP and after COI were analyzed for 27 different cytokines using a multiplex cytokine assay. In addition to CRP, 14 cytokines IL-1ra, IL-4, IL-5, IL-6, IL-8, IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, IFN-γ, IP-10, MCP-1, and MIP-1β were significantly altered ($P \leq 0.05$) during the study although some differences were low-fold elevations compared to the pre-OP levels. IL-6 as well as IL-12(p70) were consistently elevated in infected patients. Surgery influenced cytokine production with some overlap of cytokines in both groups, implying that the use of cytokines is maximized when the cytokines are not or no longer affected by surgical trauma. To lend more robustness to the selection of candidate cytokines, in addition to the statistical differences, we applied a threshold cut-off of approximately 2-fold elevations when comparisons were made. This resulted in the selection of 8 cytokines, namely IL-6, IL-1ra, IL-8, IL-12(p70), eotaxin, IP-10, MCP-1, and MIP-1β, which may be used in a multiplex assay for detection of IAI after surgery. Furthermore, IL-1ra and IL-8 may be used as prognostic cytokines prior to surgery. The present results imply that the use of cytokines may be a suitable alternative to CRP for IAI diagnosis.

1. Introduction

Implant-associated infections (IAI) still belong to the most serious complications after orthopaedic surgery and their treatment remains a challenge for the surgeon. They are caused mainly by gram-positive bacteria such as *Staphylococcus aureus* and coagulase-negative staphylococci [1,2] and they often lead to implant replacement or explantation. Moreover, IAI results in prolonged hospitalization, additional surgery, and higher costs. IAI after knee and hip arthroplasty, for instance, lead to an increase of total bed days from 7.9 to 31.6, additional procedures [3], and eventually to 3- to 5-fold higher costs than primary arthroplasty [3,4].

IAI after orthopaedic surgery can only be detected by a combination of different methods including measurement of C-reactive protein (CRP), histopathology, microbiology or imaging [5]. However, the reliability of these methods remains challenging. To improve and optimize non-invasive diagnosis of IAI, we searched for alternative biomarkers to CRP. In this respect, cytokines are a promising category of biomarkers. They are small proteins that are involved in cell signaling and are produced by various cell types including immune cells. Hence,

Abbreviations: COI, confirmation of infection; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; IAI, implant-associated infection; SS, spine surgery; TKR, total knee replacement; THR, total hip replacement

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they are especially important for immune responses such as those after bacterial infections.

The aim of this prospective study was to determine whether serum cytokine profiling can be used for diagnosis of IAI after orthopaedic surgery. The levels of CRP and 27 cytokines in serum were measured before and after orthopaedic surgery in control patients and in patients who developed an infection as well as during the infection (COI) in those infected patients.

2. Materials and methods

2.1. Study participants

Over a 1-year period, 143 patients who underwent either total knee arthroplasty (n = 41) or spine instrumentation (n = 102) were included in this prospective, single-center case-control study. Patients underwent primary and/or revision surgeries. Inclusion criteria were a minimum age of 18 years, full contractual capability, medical indication for surgery using an orthopaedic or trauma surgical implant and no infection on the day of primary surgery (clinical evaluation and CRP < 3 mg/L). All patients received intravenous general anesthesia with intubation. In addition, regional anesthesia (femoral nerve catheter) was used in 4 patients undergoing total knee arthroplasty (3 controls and 1 patient who developed an infection). For patients undergoing total knee arthroplasty, a tourniquet with a pressure of 350 mmHg was applied perioperatively for less than 2 h. Preoperatively or perioperatively, patients received an intravenous antibiotic prophylaxis with cefazolin 2 g or, in case of penicillin allergy, clindamycin 600 mg. Perioperative antibiotic prophylaxis was repeated when the duration of surgery exceeded 4 h. Patient follow-up occurred over one year after hospital discharge.

In case of IAI, at least 5 biopsies were taken from the peri-implant region and analyzed microbiologically to identify the pathogen and prove infection. If at least 2 biopsies were positive, infection was confirmed. During 1 year follow-up, 5 study participants displayed an IAI (2 with primary TKR, 1 with revision TKR and 2 with revision SS, 3 males and 2 females). The clinical features are presented in Table 1. In total, 10 patients who underwent the same surgical procedures as the infection group, but without infection, were designated as negative controls (5 with primary TKR, 4 with primary SS and 1 with revision SS, 4 males and 6 females). The demographic and clinical features of these study participants are shown in Table 2.

2.2. Blood collection and serum and plasma preparation

Blood samples were taken during hospitalization of the patients at the University Hospital of Cologne, Department of Orthopaedic and Trauma Surgery for measurement of CRP and cytokines. The times of blood draws were set according to routine hospital standards and medical needs of the patients. The timepoints (days) of blood draws for patients from the infected group are shown in Table 1. The timepoints for the controls were: patient 6 (−1, 1, 2, 6, 12, 22), patient 7 (−1, 0, 1, 2, 6, 8, 10), patient 8 (−7, 0, 1, 7, 8, 11), patient 9 (−1, 0, 1, 5, 8), patient 10 (−2, 0, 1, 4, 6, 12), patient 11 (−1, 0, 1, 2, 9), patient 12 (−4, 1, 3, 8), patient 13 (−5, 1, 3, 17), patient 14 (−2, 0, 1, 8, 11), and patient 15 (−1, 1, 6, 8); negative numbers = days pre-OP, 0 = day of surgery, and positive numbers = days post-OP. Samples for cytokine analysis were taken from a central venous catheter or from a peripheral vein of the arm and collected in serum gel tubes (S-Monovette® Serum, Sarstedt, Nümbrecht, Germany). After complete coagulation at 4 °C, samples were centrifuged for 10 min at 1,000g and 4 °C and duplicate serum samples were stored at −20 °C until analyzed. For determination of CRP, the blood was drawn as described above in lithium-heparin tubes (S-Monovette®, lithium-heparin, Sarstedt, Nümbrecht, Germany). It was centrifuged for 10 min at 4000g and 21 °C and plasma was used fresh.

Table 1
Clinical features of the 5 infected patients, as determined by microbiological analysis. Depending on the infectious agent and clinical history, the patients received antibiotic treatment.

Patient	COI (day)	Day of diagnosis and infectious agent	Period of antibiotic treatment	Duration of antibiosis (days)	Days of blood draws
1	84	d84: *S. capitis*, d105: *S. epidermidis*, d117: sterile	d84-107: Clindamycin, d107-135: Vancomycin, d135-232 Cotrimoxazol	148	−1, 1, 8, 82, 105, 109, 111, 118, 121, 173, 188, 189, 195, 197
2	18	d18: *S. epidermidis, S. haemolyticus*	d5-20: Clindamycin, d20-200: Vancomycin/Ciprofloxacin/Rifampicin	195	−2, 2, 7, 17, 18, 23, 29
3	66	d66: *S. haemolyticus, S. epidermidis*, d73: *Enterococcus faecalis, S. haemolyticus, S. epidermidis* strains I, II, *S. capitis, Finegoldia magna*	d73: Vancomycin, d76-80: Vancomycin/Ciprofloxacin/Rifampicin, d80-169: Vancomycin	96	−5, 0, 1, 5, 11, 14, 18, 21, 27, 69, 70, 74, 77, 78, 79, 85, 87, 89
4	10	d10-12: MSSA, d15: MSSA, d22: MSSA, d24: sterile	d10-14: Clindamycin, d14-42: Flucloxacillin/Rifampicin, d42-98 Clindamycin	88	−1, 0, 1, 18, 23, 25, 29, 35
5	13	d13: *P. mirabilis*, d14: *P. mirabilis, E. faecium*, d23: *P. mirabilis, E. faecium, Bacteroides fragilis*, d29: *E. faecium, P. mirabilis*	d10-26: Clindamycin, d21-26: Ciprofloxacin, d26-35: Vancomycin/Ampicillin/Sulbactam, d36-47: Metronidazol/Ciprofloxacin/Linezolid	37	−1, 1, 5, 12, 16, 19, 20, 24, 27, 28, 29, 34, 37, 46, 47, 90, 92, 94, 101, 102

S.: Staphylococcus, MSSA: methicillin-sensitive *S. aureus, E.: Enterococcus, P.: Proteus.* Days of blood draws: negative numbers = days pre-OP, 0 = day of surgery, positive numbers = days post-OP.

Table 2
Demographic and clinical features of the study participants who underwent TKR or SS. Five patients (1–5) had an IAI due to different bacterial infections. Patients 6–15 represent control patients who were not infected after surgery. Secondary diagnoses of the study participants are indicated in the last column.

Patient	Age (years)	Gender (m/f)	Type of surgery	Infection status	Secondary diagnoses
1	75	f	TKR	IAI	arterial hypertension, hypothyroidism, COPD, class II obesity, atrial fibrillation
2	83	m	TKR	IAI	arterial hypertension, COPD, diabetes mellitus type 2, essential thrombocythemia
3	70	m	TKR	IAI	arterial hypertension, hypothyroidism, class I obesity, chronic alcohol abuse, cirrhosis Child B, thrombocytopenia, microcytic hypochromic anemia, hyperuricemia
4	59	m	SS	IAI	iron deficiency anemia
5	67	f	SS	IAI	arterial hypertension, hypothyroidism, COPD, chronic polyarthritis, iron deficiency anemia
6	72	f	TKR	control	arterial hypertension, COPD, reflux esophagitis
7	70	f	TKR	control	none
8	60	m	TKR	control	arterial hypertension, class I obesity, iron deficiency anemia
9	71	m	TKR	control	arterial hypertension, chronic sinusitis maxillaris, hyperthyreosis factitia, benign prostatic hyperplasia
10	50	m	TKR	control	class I obesity
11	32	m	SS	control	class I obesity
12	76	f	SS	control	arterial hypertension, chronic kidney disease stage 2, hepatopathy
13	73	f	SS	control	arterial hypertension, class I obesity, bone metastasised breast cancer (18 years ago), iron deficiency anemia, steatosis hepatitis, pancreatic lipomatosis
14	74	f	SS	control	hypothyroidism, diabetes mellitus type 2
15	69	f	SS	control	arterial hypertension, hypothyroidism

m: male, f: female, TKR: total knee replacement, SS: spine surgery, IAI: implant-associated infection.

2.3. CRP level determination

The CRP level was determined via latex agglutination assay according to the manufacturer's instructions (C-Reactive Protein Gen.3, cobas®, Roche Diagnostics, Basel, Switzerland). Briefly, plasma was diluted 1:100 and added on a slide, which was pre-coated with antibodies to monoclonal anti-human CRP and latex reagent. After 2 min incubation, clear agglutination was observed on the slide and it was examined turbidimetrically using the analytic system cobas® C702 (Roche Diagnostics). CRP values below 3 mg/L are considered clinically irrelevant and were adjusted to 0 mg/L.

2.4. Multiplex cytokine assay

A total of 27 cytokines, namely, interleukin (IL)-1β, IL-1 receptor antagonist (ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor (FGF2), granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), IFN-γ-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, platelet-derived growth factor-bb (PDGF-bb), "Regulated on Activation, Normal T-cell Expressed and Secreted" (RANTES), tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) (Bio-Rad Laboratories, USA) were measured in duplicates in a multiplex analyzer (Bio-Plex 200®, Bio-Rad Laboratories) according to the manufacturer's instructions. Sera were thawed, centrifuged for 10 min at 10,000*g* and 4 °C and diluted in sample diluent (1:4). By using the median of the fluorescence intensity and the standard curve, the absolute concentration of the cytokines (pg/mL) was calculated (Bio-Plex Manager 6.1, Bio-Rad Laboratories).

2.5. Time intervals and sample analysis

Intervals analysed for control patients and patients who developed an infection were pre-OP as well as 0–2 and 3–12 days post-OP representing basal levels, the immediate effects of surgical trauma, and the healing process or potential infectious stage after surgery, respectively. The time of COI of patient 4 was determined at day 10 post-OP. Therefore, values for CRP and cytokines of this patient were excluded in the mean values for CRP and cytokines pre-OP and 0–12 days post-OP. During infection, however, CRP and cytokines for all 5 infected patients were analysed and compared to the pre-OP values of all 5 patients who developed an infection. Intervals analysed for the infected patients were pre-OP (all 5 patients) and 0–8 (Patient 2, 3, 4, 5), 9–13 (Patient 2, 3, 4, 5), 14–21 (Patient 1, 3, 4, 5), and 22–27 (Patient 1, 3, 4, 5) days post-COI, representing basal levels and weekly changes over a 4-week-period. Therefore, for each interval post-COI, 4 patients were included for cytokine profiling, depending on blood draws during clinical routine.

2.6. Ethics

This study was performed according to the Helsinki guidelines in compliance with national regulations for the use of human material. Utilization of human blood samples for research purposes was approved by the ethics committee of the University of Cologne (reference number 12-018). All patients gave written informed consent before participation in this study.

2.7. Statistical analyses

Differences in age and gender between the controls and the group that developed an infection were compared using the *t*-test and Fisher's exact test, respectively. Differences between the intervals post-OP or post-COI and pre-OP within the same group were assessed by the paired *t*-test whereas differences between the controls and the group that developed an infection pre-OP and post-OP were assessed by the Wilcoxon two-sample test. Fisher's exact test, the paired *t*-test, and the Wilcoxon test were carried out with the SAS procedures FREQ, TTEST, and NPAR1WAY, respectively. Values are given as the mean ± standard error of the mean (SEM). P-values ≤ 0.05 were considered statistically significant. For statistical analyses, GraphPad Prism 7 (La Jolla, CA, USA) and SAS/STAT software 9.4 (SAS Institute Inc: SAS/STAT User's Guide, Cary NC: SAS Institute Inc, © 2002–2012) were used.

3. Results

The median duration of surgery in control patients (n = 10) and those who developed an infection (n = 5) was 162.5 (range 95–385) and 138 (range 105–272) min, respectively. Three cytokines were below the lower limit of quantification for most samples (GM-CSF < 2.18 ± 0.95 pg/mL, IL-1β < 1.75 ± 0.01 pg/mL, and IL-15 < 2.26 ± 1.23 pg/mL) and were excluded from further analyses (data not shown). The cytokines IL-2, IL-5, IL-10, and MIP-1α were not analysed pre- and post-OP in the patients belonging to the group that

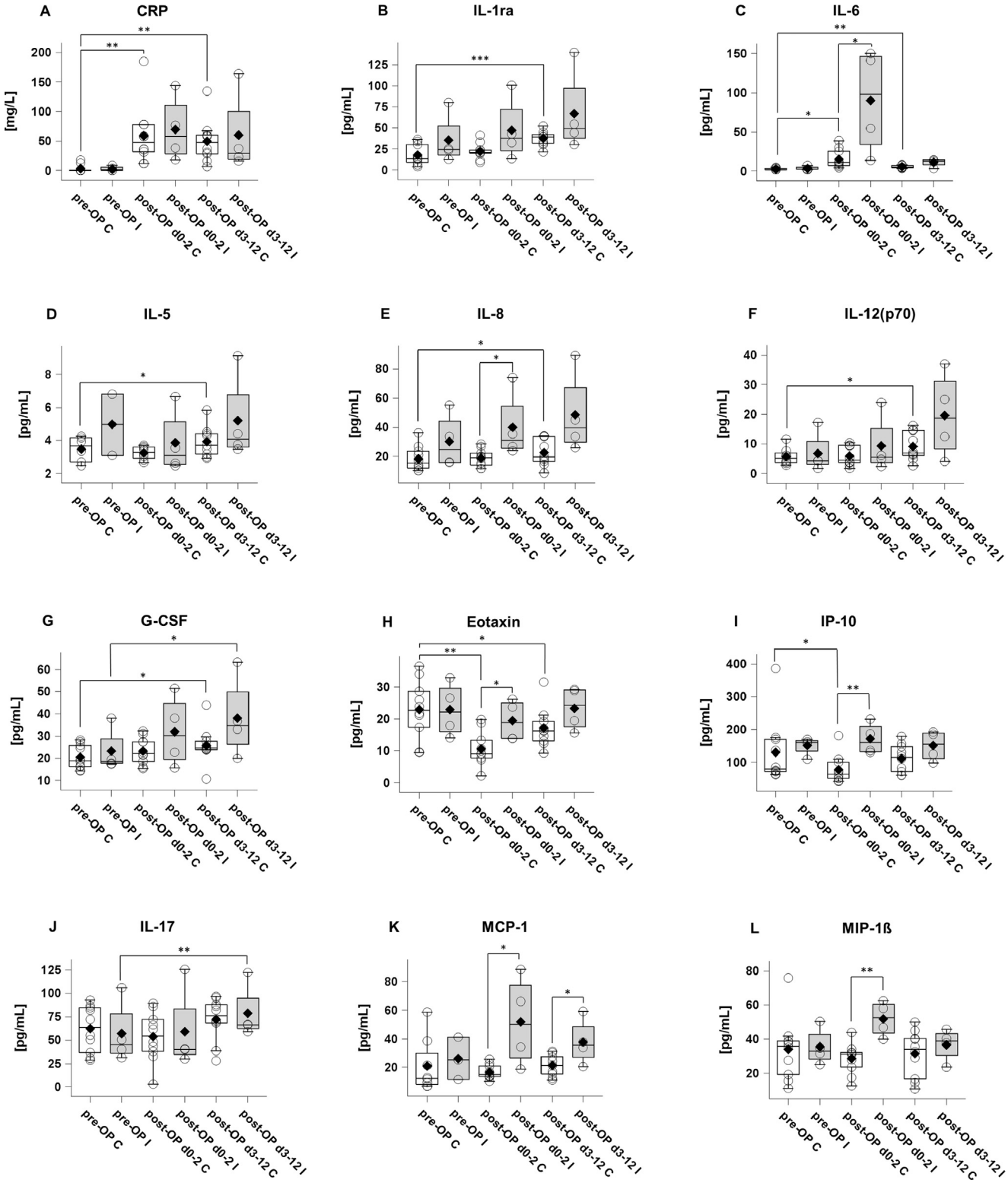

Fig. 1. A–L: Altered serum CRP and cytokine levels pre- (pre-OP) and post-surgery (post-OP) in control (n = 10, white bars) and infected patients (n = 4, grey bars). Samples were taken pre-OP and 0–2 and 3–12 days post-OP. The absolute CRP concentration in mg/L and cytokine concentrations in pg/mL are indicated on the y-axis. Box-and-whiskers plot; data points: open circles, maximum: endpoint of upper whisker, minimum: endpoint of lower whisker, third quartile (75th percentile): upper edge of box, first quartile (25th percentile): lower edge of box, median (50th percentile): line inside box, mean: ♦, data points beyond the whiskers: outliers. Results are expressed as mean ± SEM, and significant differences in the serum levels ≤ 0.05 are highlighted by asterisks: *P ≤ 0.05; **P < 0.01, ***P < 0.001.

developed an infection due to out of range data or values that were not reliable (outliers), which resulted in values for less than 3 patients in at least one interval post-OP. For the 5 infected patients, all 24 cytokines measured except IL-5 were analysed for the post-COI interval. No significant differences were observed in age and gender between controls and the patients who developed an infection (data not shown). In total, 7 out of 20 cytokines (IL-7, IL-9, FGF2, PDGF-bb, RANTES, TNF-α, and VEGF) in controls, patients who developed an infection and

infected patients pre-OP, post-OP, and post-COI did not show any significant changes (data not shown).

3.1. In control patients, compared to pre-OP levels, CRP, IL-1ra, IL-6, IL-5, IL-8, IL-12(p70), and G-CSF increased significantly while eotaxin and IP-10 decreased significantly after orthopaedic surgery

In control patients, the CRP level increased significantly from pre-OP to 0–2 days (18.5-fold) and 3–12 days post-OP (15.6-fold) (Fig. 1A). In the interval 0–2 days post-OP, significant increases were observed for IL-6 (5.4-fold, Fig. 1C) while significant decreases were found for eotaxin (2.1-fold, Fig. 1H) and IP-10 (1.7-fold, Fig. 1I). There were significant increases 3–12 days post-OP in IL-1ra (2.1-fold, Fig. 1B), IL-6 levels (1.9-fold, Fig. 1C), IL-5 (1.1-fold, Fig. 1D), IL-8 (1.2-fold, Fig. 1E), IL-12(p70) (1.6-fold, Fig. 1F), and G-CSF (1.3-fold, Fig. 1G). Eotaxin levels were still significantly reduced 3–12 days post-OP (1.3-fold, Fig. 1H). Peak values were observed pre-OP for eotaxin, IL-9, IP-10, MIP-1β, and RANTES, 0–2 days post-OP for CRP and IL-6, and 3–12 days post-OP for the remaining 18 cytokines.

3.2. In patients who developed an infection, compared to pre-OP levels, G-CSF and IL-17 levels increased significantly after orthopaedic surgery

In patients who developed an infection, compared to basal levels, CRP levels increased 23.3-fold 0–2 days post-OP ($P = 0.10$) and exhibited a 20-fold increase 3–12 days post-OP ($P = 0.21$) (Fig. 1A). At 3–12 days post-OP, significant increases were observed for G-CSF (1.6-fold, Fig. 1G) and IL-17 (1.4-fold, Fig. 1J). IL-6 levels showed a 24.6-fold increase 0–2 days post-OP ($P = 0.08$) and a 3.1-fold increase 3–12 days post-OP ($P = 0.07$), (Fig. 1C). Peak values were observed pre-OP for none of the cytokines, 0–2 days post-OP for CRP, IL-2, IL-6, IP-10, MCP-1, and MIP-1β, and 3–12 days post-OP for the remaining 19 cytokines.

3.3. Compared to controls, patients who developed an infection had significantly higher levels of IL-6, IL-8, eotaxin, IP-10, MCP-1, and MIP-1β after orthopaedic surgery

We also compared CRP and cytokine levels of control patients and patients who developed an infection 0–2 and 3–12 days post-OP. CRP levels were not significantly different between controls and patients who developed an infection (Fig. 1A). In patients who developed an infection, significantly higher values were detected 0–2 days post-OP for IL-6 (5.7-fold, Fig. 1C), IL-8 (2.1-fold, Fig. 1E), eotaxin (1.8-fold, Fig. 1H), IP-10 (2.2-fold, Fig. 1I), MCP-1 (3.1-fold, Fig. 1K), and MIP-1β (1.8-fold, Fig. 1L). In addition, at 3–12 days post-OP, MCP-1 levels were 1.8-fold significantly higher in patients who developed an infection than in the controls (Fig. 1K).

3.4. In infected patients, CRP, IL-6, IL-4, IL-13, IL-8, IL-12(p70), and IFN-γ levels increased significantly post-COI

To determine the CRP and cytokine profiles during IAI, we examined their levels at 4 intervals post-COI (d0-8, d9-13, d14-21, and d22-27) and compared them with the basal levels of the infected patients; day 0 being the day of confirmation of infection. CRP levels increased significantly 20.5-fold 0–8 days post-COI and remained elevated until 22–27 days post-COI (9.4-fold increase, Fig. 2A). Significant increases were observed at 9–13 days post-COI for IL-4 (1.3-fold, Fig. 2C), IL-13 (1.3-fold, Fig. 2D), IL-8 (1.4-fold, Fig. 2E), and IL-12(p70) (3.3-fold), at 14–21 days post-COI for IL-6 (2.4-fold, Fig. 2B) ($P = 0.05$), and at 22–27 post-COI for IL-12(p70) (1.7-fold, Fig. 2F) and IFN-γ (1.2-fold, Fig. 2G). Peak values were observed pre-OP for eotaxin, IL-5, MIP-1β, and RANTES, 0–8 days post-COI for CRP, and 9–13 days post-COI for the remaining 20 cytokines.

3.5. Candidate cytokines for IAI diagnosis

As observed for the above-mentioned results, some of the differences in the cytokine levels were significant but showed low-fold elevations compared to basal levels (pre-OP levels) and *vice versa*. To increase the robustness in selection of candidate cytokines, a minimum threshold fold-elevation can be implemented. As such, we suggest that a 2-fold elevation should be used in addition to the significant differences. Based on this approach, the candidate cytokines are shown in Table 3. Although the levels of 6 cytokines, namely IL-4, IL-5, IL-13, IL-17, G-CSF, and IFN-γ were significantly different from basal levels their elevations did not meet the designated threshold level. Thus, the remaining 8 cytokines IL-6, IL-1ra, IL-8, IL-12(p70), eotaxin, IP-10, MCP-1, and MIP-1β should be considered as candidate cytokines for IAI diagnosis.

4. Discussion

To date, there is still a lack of methods to diagnose IAI, which are both sensitive and specific. However, it is of paramount importance to obtain an early reliable diagnosis to commence antibiotic treatment and, if necessary, revision surgery. Serum markers could function as suitable diagnostic tools since blood can be easily drawn, especially during routine surgical and follow-up procedures, which present a low risk to patients. To our knowledge, this is the first prospective study in which a broad spectrum of 27 cytokines was analysed via multiplex assay using sera from patients with and without an IAI that were obtained pre-OP, post-OP, and during the course of infection. Multiplex cytokine assay using serum samples is a non-invasive method, whereby the results are obtained within hours.

In the present study, consistently high elevations in CRP were observed in all phases in both groups of patients after surgery and in infected patients. Thus, our results show that CRP is not a reliable diagnostic marker for IAI when CRP is elevated. As an alternative for IAI diagnosis, we evaluated the elevations in cytokines to select a group of candidate cytokines, which may be used in a multiplex assay with a larger cohort of patients. The results of our study show that, in addition to CRP, 14 different cytokines IL-1ra, IL-4, IL-5, IL-6, IL-8, IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, IFN-γ, IP-10, MCP-1, and MIP-1β were significantly altered during the study although some differences were low-fold elevations compared to the pre-OP levels. Notably, surgery influenced cytokine production with some overlap of cytokines in both groups. Compared to pre-OP values, elevations in IL-6 and a decrease in eotaxin 0–2 days post-OP indicate responses to surgery-associated sterile inflammation, as shown in the controls. The results imply that the use of cytokines is maximized when the cytokines are not or no longer affected by surgical trauma, which may vary according to the cytokines measured. To lend more robustness to the selection of candidate cytokines, in addition to the statistical differences, we also applied a threshold cut-off of 2-fold (including 1.8-fold) elevations when comparisons were made. This approach led to the reduction of the number of cytokines to a total of 8, namely IL-6, IL-1ra, IL-8, IL-12(p70), eotaxin, IP-10, MCP-1, and MIP-1β. These candidate cytokines may be used in a multiplex assay for detection of IAI. Based on the comparison between the pre-OP levels in the controls and patients that developed an infection only 2 of these cytokines, that is, IL-1ra and IL-8 may be used as prognostic cytokines prior to surgery. For diagnostic work, all 8 cytokines may be measured after surgery. Since the immune system and therefore, cytokine levels of each patient is unique and may differ from each other, baseline values may provide additional information for prognostic and diagnostic work, as performed in the present study. The pre-OP values may be used to compare elevations at timepoints after surgery. Taking this approach into consideration, the minimum of 4 cytokines IL-6, IL-1ra, IL-12(p70), and MCP-1 should be assayed during routine clinical work. Furthermore, it should be noted that only IL-6 and IL-12(p70) were consistently elevated in infected

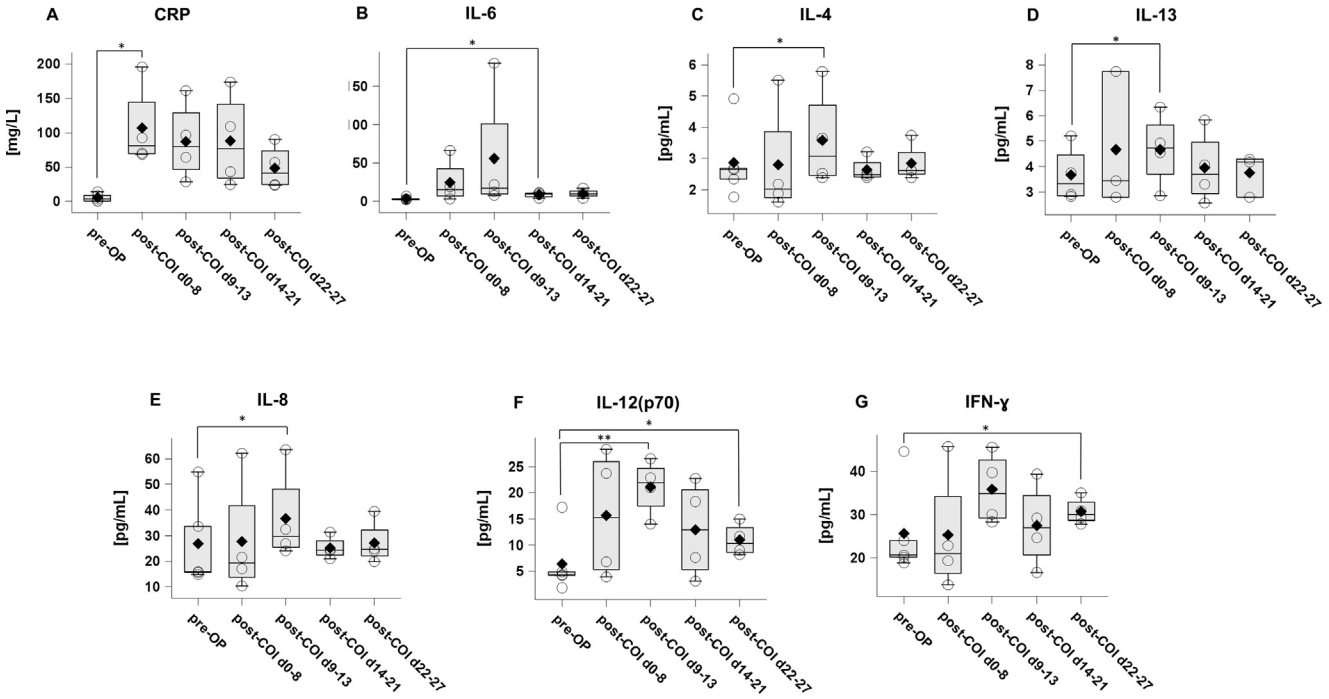

Fig. 2. A–G: Altered serum CRP and cytokine levels post-COI. Samples were taken pre-OP and 0–8, 9–13, 14–21, and 22–27 days post-COI (n = 5 patients). The absolute CRP concentration in mg/L and cytokine concentrations in pg/mL are indicated on the y-axis. Box-and-whiskers plot; data points: open circles, maximum: endpoint of upper whisker, minimum: endpoint of lower whisker, third quartile (75th percentile): upper edge of box, first quartile (25th percentile): lower edge of box, median (50th percentile): line inside box, mean: ♦, data points beyond the whiskers: outliers. Results are expressed as mean ± SEM, and significant differences in the serum levels ≤ 0.05 are highlighted by asterisks: *P ≤ 0.05; **P < 0.01.

Table 3
Summary of cytokines in fold-elevation according to the different timepoints.

	Pre-OP I vs. C	Post-OP C vs. pre-OP C			Post-OP I vs. pre-OP I			Post-OP I vs. C			Post-COI vs. pre-OP I				
		0–2	3–12		0–2	3–12		0–2	3–12		0–8	9–13	14–21	22–27	
CRP	–	x	x	*x*	–	–	*x*	–	–		x	–	–	–	*x*
	0.9	18.5	15.7		23.3	20		1.2	1.2		20.5	16.8	16.9	9.4	
IL-6	–	x	x	*x*	–	–	*x*	x	–	*x*	–	–	x	–	*x*
	1.3	5.4	1.9		24.6	3.1		5.7	2.0		7.0	15.6	2.4	2.9	
IL-1ra	–	–	x	*x*	–	–	*x*	–	–	*x*	–	–	–	–	
	2.0*	1.3	2.1		1.3	1.9		2.1	1.8		1.0	1.5	−1.1	−1.2	
IL-8	–	–	x		–	–		x	–	*x*	–	x	–	–	
	1.6£	1.0	1.2		1.3	1.6		2.1	2.2		1.0	1.4	−1.1	1.0	
IL-12(p70)	–	–	x		–	–	*x*	–	–	*x*	–	x	–	x	*x*
	1.2	1.1	1.6		1.4	2.9		1.6	2.2		2.4	3.3	2.0	1.7	
Eotaxin	–	x	x	*x*	–	–		x	–	*x*	–	–	–	–	
	1.0	−2.1	−1.3		−1.2	1.0		1.8	1.4		−1.5	−1.2	−1.3	−1.1	
IP-10	–	x	–		–	–		x	–	*x*	–	–	–	–	
	1.2	−1.7	−1.2		1.1	1.0		2.2	1.3		−1.1	−1.3	1.1	1.1	
MCP-1	–	–	–		–	–	*x*	x	x	*x*	–	–	–	–	
	1.2	−1.1	1.1		2.0	1.5		3.1	1.8		−1.4	1.1	−1.9	−1.4	
MIP-1β	–	–	–		–	–		x	–	*x*	–	–	–	–	
	1.0	−1.2	−1.1		1.5	1.0		1.8	1.2		−1.2	1.0	−1.1	−1.1	
Candidate cytokines for each timepoint	*IL-1ra** *IL-8£*	CRP IL-6 eotaxin	CRP IL-6 IL-1ra		CRP IL-6 MCP-1	CRP IL-6 IL-1ra IL-12(p70) MCP-1		IL-6 IL-1ra IL-8 eotaxin IP-10 MCP-1 MIP-1β	IL-6 IL-1ra IL-8 IL-12(p70) MCP-1		CRP IL-6 IL-12(p70)	CRP IL-6 IL-12(p70)	CRP IL-6 IL-12(p70) MCP-1	CRP IL-6 IL-12(p70)	
Candidate cytokines for total period	*IL-1ra**, *IL-8£*	CRP, IL-6, IL-1ra, eotaxin			CRP, IL-6, IL-1ra, IL-12(p70), MCP-1			IL-6, IL-1ra, IL-8, IL-12(p70), eotaxin, IP-10, MCP-1, MIP-1β			CRP, IL-6, IL-12(p70)				

Cytokines shown in the table were statistically significant and/or showed at least a 2-fold-elevation at a minimum of one timepoint during the study.
I: infected patients, C: control patients, x: statistically significant; -: not statistically significant, *x*: candidate cytokines.

* P = 0.3580.

£ P = 0.2293.

patients.

In the literature, there is only one report on the measurement of a large spectrum of cytokines with 48 healthy patients after knee or hip arthroplasty (n = 25 cytokines) [6] and another with THR in 20 patients (n = 30 cytokines) [7]. Furthermore, in these two studies, there are contradictions concerning the number of cytokines up-regulated or down-regulated, which may be due to various factors including the patient cohorts.

The pro-inflammatory IL-6 is produced mainly by macrophages but also by monocytes, fibroblasts, and T2 lymphocytes after trauma. It transcriptionally regulates the production of CRP in the liver [8,9]. For diagnosing periprosthetic infections, Di Cesare et al. [10] and Gollwitzer et al. [11] showed that serum IL-6 could be used as a marker. In another study, one patient with a deep infection showed increased levels of IL-6 6 h post-OP [6]. Interestingly, in the present study, the magnitude of IL-6 elevations in patients who developed an infection was 24.6 fold-higher than pre-OP values and 5.7-fold higher than that in the control group 0–2 days post-OP. IL-6 levels showed 7–15.6-fold elevations for the period 0–8 and 9–13 days post-COI. Although IL-6 was also affected by surgery, compared to pre-OP levels, elevations were considerably higher 0–2 days and decreased 3–12 days post-OP. Therefore, IL-6 should be considered as a candidate cytokine for IAI diagnosis based on the magnitude of its elevation.

Previous reports compared the levels of CRP and/or a limited number of cytokines including IL-6 for diagnosis of infection. Using a combination of both IL-6 and CRP, the sensitivity was 0.57–1 and the specificity ranged from 0.86 to 1 [12–14]. IL-6 and CRP increased significantly in patients with septic loosening of THR [15] and distinguished between patients with and without postoperative orthopaedic joint prosthesis infections (TKR and THR) [16]. Serum IL-6 and CRP are effective for excluding while IL-6 is also effective for confirming prosthetic joint infection [17]. Furthermore, CRP and IL-6 were reported to be suitable biomarkers for determining late-onset chronic (low-grade) IAI [18].

Since both CRP and some cytokines are produced in response to anesthesia, surgical trauma, inflammation, and infection optimal threshold values may be used to diagnose prosthetic joint infection (PJI). For example, CRP cut-off levels were set at 3.2–23.5 mg/L [19–29]. IL-6 cut-off levels were set at 2.6–13350 pg/mL [10,14,24,26,30]. Bottner et al. [12] also reported that all deep infections can be recognized by a combination of CRP (> 3.2 mg/dL) and IL-6 (> 12 pg/mL). However, these levels may have a broad range and vary according to various factors including the individual patients. Therefore, increments in cytokine levels expressed as fold-elevation compared to basal levels, as practiced in our study, would be useful instead of considering the levels alone. For example, in synovial fluid, IL-6 showed a 27-fold elevation and CRP a 13-fold elevation for diagnosing PJI [26]. Furthermore, an increase and decrease in cytokine levels followed by a repeated increase, that is, a bimodal pattern [31] would indicate that there were alterations in the health of the patient.

IL-1ra is an IL-1 antagonist and therefore protects against the pro-inflammatory effects of IL-1. In this study, a significant 2.1-fold elevation in IL-1ra was observed exclusively in the control group 3–12 days post-OP. In patients who became infected, IL-1ra increased 1.3-fold 0–2 days post-OP and 1.9-fold 3–12 days post-OP. Notably, we were not able to measure IL-1β levels in the present study. IL-1ra was reported by other groups to be up-regulated directly post-OP [32] and before IL-6 elevation [33,34]. There was an increase in IL-1ra for at least 7 days after spinal instrumentation [35], in hip-fracture-operated elderly patients [36] and in both infected and non-infected patients undergoing revision TKR or THR [37]. In contradiction to our results and the above-mentioned reports, a significant decrease in IL-1ra was observed by Reikeras et al. [7] while Shah et al. [6] reported no alterations in this cytokine. IL-1ra showed a significant reduction systemically but local levels from drained wound blood were 20- to 30-fold higher 24 h post-OP [38]. It should therefore be expected that IL-1ra generally increases after surgery.

The pro-inflammatory cytokine IL-8 is also one main mediator of acute inflammation, which activates neutrophils and stimulates angiogenesis [34]. A strong increase in IL-8 in PJI after TKR and THR was reported [37]. There were significant elevations in IL-8 in synovial fluid from patients with a PJI after THR or TKR [26] and it was detected directly in synovial fluid of IAI patients [39].

In contrast to the present study, no significant increases were observed in IL-12 concentrations after surgery [38,40] or in patients with PJI [37]. Reikeras et al. observed a significant decrease in IL-12 within 6 days after surgery compared to preoperative values. These contradictory reports may be due to the variation in the duration of the studies, but they implicate that elevation in IL-12 may indicate the presence of an infection.

To our knowledge, there are very few reports on alterations in eotaxin after orthopaedic surgery and none from infected patients. Eotaxin is a small chemokine that selectively recruits eosinophils and has pro-inflammatory effects. In our study, we observed decreased eotaxin levels in the controls at day 0–2 and 3–12 post-OP while at day 0–2 post-OP, eotaxin levels were higher in patients who became infected than in controls. A decrease in eotaxin over the 6-day post-operative course was also observed by Reikeras et al. [7] but not by Shah et al. [6]. In the present study, eotaxin levels post-COI were 1.1–1.5-fold lower than those pre-OP. These data imply that generally eotaxin levels decrease both after surgery and infection.

There are very few reports about alterations in the pro-inflammatory chemokine IP-10 after surgery and none during infection. In the present study, while a significant 1.7-fold decrease was observed in the control group 0–2 days post-OP, the level of IP-10 was significantly 2.2-fold higher in the group that became infected compared to controls 0–2 days post-OP. No significant alterations in IP-10 were observed after surgery by Shah et al. [6] and Reikeras et al. [7] and post-COI in the present study.

MCP-1 is a pro-inflammatory cytokine, which decreased 6 h post-OP in patients who underwent hip and uni-compartmental arthroplasty of the hip [6]. In contrast, in the same study, one patient with a deep infection had even lower MCP-1 levels 6 h post-OP but higher IL-6 levels compared to the healthy control patients, leading the authors to the suggestion that these changes in IL-6 and MCP-1 might reflect inflammatory events [6]. In the present study, MCP-1 levels were significantly 1.8–3.1-fold higher in patients who later became infected compared to controls. Neither significant changes in MCP-1 after surgery [7] nor significant elevations in synovial fluid from patients with a PJI after THR or TKR [26] were reported.

MIP-1β synthesis is stimulated by bacterial endotoxins and is known for its chemotactic and pro-inflammatory effects [41]. Non-significant changes were reported after surgery [6,7]. In contrast, significant elevations in synovial fluid from patients with a PJI after THR or TKR were observed [26]. In the present study, although there were no significant alterations within the groups, MIP-1β levels were significantly 1.8-fold higher in patients who later became infected compared to controls.

In this study, 14 out of 15 patients suffered from at least one disease, the most frequent ones being arterial hypertension (10/15), hypothyroidism (5/15), class I obesity (5/15) and COPD (4/15). The effect of comorbidities on the cytokine levels was not determined in this study but it should be noted that the distribution of diseases in both study groups was comparable.

Besides the influence of anesthesia, surgical trauma, and comorbidities on cytokine levels, there are limitations to our study. Firstly, only 3.5% of the initial 143 patients who underwent orthopaedic surgery were diagnosed with IAI. Low infection rates are expected and are preferable in the clinic. However, to establish cytokines as biomarkers for IAI diagnosis, a follow-up study with a larger cohort of infected patients is needed including the 8 candidate cytokines IL-6, IL-1ra, IL-8, IL-12(p70), eotaxin, IP-10, MCP-1, and MIP-1β. Furthermore, prophylactic pre- and perioperative antibiotic treatment,

which all patients in our study received, may also influence cytokine levels [42]. However, due to the known benefit, omitting the standard perioperative antibiotic prophylaxis would not be possible. Individual variations of the CRP and cytokine profiles may be high due to secondary diagnoses, necessitating the collection of a blood sample prior to surgery for determination of individual baseline values, as was performed in this study. Notably, for routine clinical work, ideally biomarkers are needed, which are applicable to all patients at all timepoints.

Author contributions

Conceived and designed the experiments: KZ, RH, GS, EM. Performed the experiments: KZ, RH. Analyzed the data: EM. Contributed reagents/materials/analysis tools: KZ, EM. Wrote the paper: KZ, RH, EM. All authors approved the submitted version.

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Competing interests

The authors declare no conflict of interests.

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Appendix A. Supplementary material

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